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Multiple-Locus Variable-Number Tandem-Repeat Analysis for Longitudinal Survey of Sources of *Pseudomonas aeruginosa* Infection in Cystic Fibrosis Patients▽†

Hoang Vu-Thien,[1] Gaëlle Corbineau,[2] Katia Hormigos,[2] Brigitte Fauroux,[3] Harriet Corvol,[3] Annick Clément,[3] Gilles Vergnaud,[2,4] and Christine Pourcel[2]*

Bactériologie[1] and Pneumologie Pédiatrie,[3] Hôpital Armand Trousseau, Assistance Publique-Hôpitaux de Paris (APHP), INSERM UMR S-719, 75012 Paris, France; Univ Paris-Sud, Institut de Génétique et Microbiologie, Orsay F-91405, France, and CNRS, Orsay F-91405, France[2]; and Division de Microbiologie Analytique, Centre d'Etudes du Bouchet, Vert le Petit, France[4]

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In order to identify the source of infection by *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF), systematic genotyping of isolates is necessary. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) was used to survey the sources of *P. aeruginosa* infections in a French (Paris, France) pediatric CF center. Between January 2004 and December 2006, 108 patients ages 2 to 21 years who were regularly monitored at the center provided sputum for culture. *P. aeruginosa* was detected in 46 children, 17 of whom had primary colonization. A total of 163 isolates were recovered. MLVA was improved from a previously published method by the addition of new, informative, and easily typeable markers. Upon genotyping with 15 VNTRs, a total of 39 lineages composed of indistinguishable or closely related isolates, were observed. One of them corresponds to "clone C," which is widely distributed in Europe, and another corresponds to reference strain PA14. Six patients were colonized with two different strains, and the remaining 40 patients were colonized with a single strain. Strains from seven lineages were shared by at least two and up to four patients among a total of 20 patients. The study demonstrates that MLVA is an efficient, easy, and rapid molecular method for epidemiological surveillance for *P. aeruginosa* infection. The resulting data and strain genetic profiles can be queried on http://bacterial-genotyping.igmors.u-psud.fr.

Cystic fibrosis (CF) is caused by a mutation in the *cftr* gene, which leads to the dysfunction of the exocrine glands. The disease is responsible for chronic obstruction in the lung, a condition favorable for *Pseudomonas aeruginosa* pulmonary infections during childhood. Aggressive antimicrobial treatment may eradicate *P. aeruginosa* at an early stage, but at a later stage chronic colonization is frequently observed (11). Chronically infecting bacteria produce alginate and form biofilms, which provide physical protection against antimicrobial treatment and which are associated with antibiotic resistance (9, 23).

P. aeruginosa is widely distributed in the environment, and it is not clear how the patients become infected. Although cross-infection between unrelated CF patients attending the same health center is believed to be uncommon, several studies have shown that some clones may be responsible for the infection of multiple patients (1, 5, 26, 37). However, it is not known whether these clones are more pathogenic than those isolated sporadically (34). Although most patients harbor a single genotype, some patients are colonized with multiple genotypes (41). A study by Munck et al. has shown that efficient and early antibiotic therapy allowed the elimination of the organisms responsible for primary colonization and that chronic infection was caused by another strain (25).

In order to determine the origin of primary colonization and to detect infections in several patients with the same strain, it is necessary to genotype isolates sequentially. Ideally, all the isolates recovered from CF patients should be genotyped to allow epidemiological surveillance of this infection. The availability of molecular methods for genotyping with a high degree of discriminatory power has opened the way to epidemiological studies. The most frequently used techniques are pulsed-field gel electrophoresis (PFGE) and ribotyping, which remain relatively expensive and time-consuming (3, 12, 39). Despite improvements in the protocol, in many laboratories PFGE is not being used on a routine basis for the genotyping of *P. aeruginosa* (10).

Other techniques use repetitive-element-based PCR and are simple and fast, but they lack interlaboratory reproducibility (37). The use of these techniques has made it possible to investigate local *P. aeruginosa* isolates within a center, but intercenter comparisons are almost impossible. A multilocus sequence typing scheme which could be a portable technique was developed, but its cost makes it impossible for use in small laboratories on a routine basis (7).

There is thus a need for a highly informative genotyping method which could be applied to a large number of samples at a low cost and which would allow interlaboratory comparisons. Multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA) is now widely used for the genotyping of many different pathogenic bacteria (18, 30, 42). Each strain is described by a code corresponding to the number of repeats at

* Corresponding author. Mailing address: Institut de Génétique et Microbiologie, Bât 400, Université Paris-Sud, 91405 Orsay cedex, France. Phone: 33 1 69 15 30 01. Fax: 33 1 69 15 66 78. E-mail: christine.pourcel@u-psud.fr.

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the selected VNTR. These codes can be easily compared between laboratories through web-based databases (WebServices), such as the one available at http://bacterial-genotyping.igmors.u-psud.fr (21). We previously described a small collection of VNTR markers for the typing of *P. aeruginosa* and showed that the clustering achieved by MLVA was similar to that achieved by ribotyping and PFGE (27). We have now added new markers to the genotyping scheme and improved the PCR amplification, which make the assay more robust and significantly increase the amount of information provided. With the present longitudinal study of *P. aeruginosa* isolates from children with CF, we demonstrate that MLVA constitutes a very robust method for the epidemiological surveillance of *P. aeruginosa* infections.

MATERIALS AND METHODS

Patients. As a control group, *P. aeruginosa* isolates were recovered between June 2004 and March 2005 from 15 patients with different diseases from the intensive care unit (ICU; the ICU group) of the Armand Trousseau Hospital. The CF patients attended a CF center in another building of the same hospital. The criterion used for the diagnosis of CF was either the presence of two mutations in the *cftr* gene or one or no mutation in *cftr* in association with a positive sweat test, defined by a chloride (Cl^-) ion concentration greater than 60 mmol/liter. Sputum samples were collected from the lower airways during an outpatient consultation or hospitalization. Forty-six of the 108 patients (ages, 2 to 21 years) attending the CF center (the CF group) during the course of this study (January 2004 to December 2006) were found to harbor a *P. aeruginosa* strain ($>10^2$ CFU/ml sputum), and for 17 of these patients it corresponded to a primary colonization (see Table S1 in the supplemental material). Among these patients, a past infection could not be totally excluded for three patients.

Microbiology. Sputum samples were inoculated onto sheep blood agar and cysteine, lactose, electrolyte-deficient agar (Oxoid, Dardilly, France). After 2 days of incubation at 35°C, colonies that looked nonidentical were picked and tested for an oxidase reaction. The biochemical identification of *P. aeruginosa* species was performed by standard techniques, and antimicrobial susceptibility was performed by disk diffusion. By using the routine O-polysaccharide typing system, mucoid strains (PMU) were usually nonserotypable (polyagglutinable), especially for the patients chronically colonized with *P. aeruginosa*. Among the nonmucoid strains, many strains were nontypeable (PNT). Different isolates from a single patient sample were recovered when they showed different serotypes or phenotypes (mucoid or nonmucoid) or when more than one difference in the antimicrobial susceptibility pattern (22 antibiotics) was observed. Isolates were preserved at −80°C in brain heart broth (bioMerieux, Marcy l'Etoile, France) with 10% glycerol (Sigma-Aldrich, Saint-Quentin Fallavier, France).

A total of 24 isolates from ICU patients and 163 isolates from CF patients were analyzed. In addition, 50 isolates from non-CF patients that had previously been genotyped by MLVA with six VNTRs and that were representative of the major groups described by Onteniente et al. (27) were analyzed with the new VNTRs.

Strains C50 (a clinical isolate) and SG17M (an environmental isolate) belonging to the widely distributed "clone C" were a generous gift from Ute Römling (Stockholm, Sweden) (34).

Reference strain PAO1 was purchased from the Institut Pasteur culture collection. *Alcaligenes xylosoxidans*, *Burkholderia cepacia*, and *Stenotrophomonas maltophilia* (one, two, and six isolates, respectively) were isolated from the sputum of CF patients. Twelve DNA samples from seven different *Pseudomonas* species (two *P. poae* isolates, one *P. tolaasii* isolate, one *P. trivialis* isolate, three *P. graminis* isolates, two *P. orientalis* isolates, two *P. fluorescens* isolates, and one *P. putida* isolates) were provided by the Centre d'Etudes du Bouchet's collection of environmental strains and related genomic DNA resources (Vincent Ramisse, Vert le Petit, France).

DNA purification. DNA was purified with a DNeasy tissue kit (QIAGEN, Courtaboeuf, France) or, alternatively, by the classical cetyltrimethylammonium bromide (CTAB)-phenol extraction method. Bacteria were lysed into a solution containing 10 mM Tris HCl, pH 8, 10 mM EDTA, 10 mM NaCl, and 0.5% sodium dodecyl sulfate and were incubated overnight at 37°C with 100 μg/ml proteinase K. One hundred microliters of 5 M NaCl was added to 0.6 ml lysate (final concentration, 0.7 M), followed, after homogenization, by the addition of 40 μl of 10% CTAB in 0.7 M NaCl (final CTAB concentration, 0.5%). After 10 min at 65°C, the CTAB precipitate was extracted with 1 volume of chloroform, and the supernatant was transferred into a fresh tube. The DNA was purified by three successive extractions with phenol (pH 7.5), phenol-chloroform (1/1), and chloroform. The nucleic acids were precipitated with 2 M NaCl and 2 volumes of ethanol. After centrifugation the pellet was suspended in TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA). The quality and the concentration of DNA were measured with an ND-1000 spectrophotometer (NanoDrop; Labtech, Palaiseau, France).

Genotyping. In addition to the markers described by Onteniente et al. (27), 11 new polymorphic tandem repeats were identified in the sequenced genomes of reference strains PAO1 and PA14 by using the strain comparison tool developed by Denoeud and Vergnaud (8), available at http://minisatellites.u-psud.fr/ (Table 1). Oligonucleotide primers targeting the 5′ and 3′ flanking regions of the VNTR loci were used for amplification. PCRs were performed in reaction mixtures of 15 μl containing 5 to 10 ng of DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 1 U of *Taq* DNA polymerase (QIAGEN), 200 μM each deoxynucleoside triphosphate, and 0.3 μM each flanking primer (Eurogentec, Angers, France) in the presence of 0.5 M betain, as described previously (14). Amplification was performed with a PTC 200 thermocycler (Bio-Rad, Marnes-la-Coquette, France) under the following conditions: an initial denaturation cycle for 5 min at 94°C and 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and elongation for 45 s at 72°C, plus a final elongation step for 10 min at 72°C. For the analysis of all markers except ms207 and ms209, 2 μl of each of the PCR products was separated in a 2% agarose gel (routine-use grade; Eurogentec, Angers, France). Electrophoresis was performed in 20-cm-wide gels made in 0.5× TBE (Tris-borate-EDTA) buffer (Sigma) and run at 8 V/cm. Reference strain PAO1 was included in each PCR run. The 100-bp ladder DNA size marker was from MBI Fermentas (Euromedex, Souffelweyersheim, France). After the run the gels were stained in 0.5 to 1.0 μg/ml ethidium bromide for 15 to 30 min and then rinsed with water and photographed under UV illumination (Vilber-Lourmat, Marne la Vallée, France).

The size of each amplicons was measured with BioNumerics (version 4.6) software (Applied-Maths, Sint-Martens-Latem, Belgium), and the number of repeats was deduced by using the PAO1 sequence as a reference. An automated sequencer (CEQ 8000; Beckman Coulter, Roissy, France) was used to analyze markers ms207 and ms209 (6-bp repeat unit) with the size marker recommended by the manufacturer. Alternatively, for these microsatellites, 4% agarose gels made of 2% standard agarose plus 2% MetaPhor (FMC BioProducts, TEBU, Le Perray en Yvelines, France) were used with a 20-bp ladder size marker from MBI Fermentas.

To help with the conversion of the allele size in base pairs into the number of repeats, we propose on a dedicated website (http://bacterial-genotyping.igmors.u-psud.fr/pseudomonas/) a chart showing the different alleles observed in our collection of more than 200 isolates. This chart will be updated when new alleles are encountered.

Nomenclature and description of MLVA profiles. The repeat length and the number of repeat units in the different sequenced genomes were determined by using the Microbial Tandem Repeats Database (http://minisatellites.u-psud.fr) (8, 22). The amplification of DNA from reference strain PAO1 with the primers described in Table 1 produced amplicons of the expected sizes. The number of repeats in new alleles was estimated by subtracting the invariable flanking region from the amplicon size and then dividing by the repeat unit length determined for reference strain PAO1. Intermediately sized alleles (which may result from intermediately sized repeat units or from small deletions in the flanking sequence) were reported as half sizes, when they were observed. Alleles of unexpected size were sequenced, and the sequences have been deposited in the EMBL sequence database. The "NA" (not amplified) designation was given when no amplification was repeatedly observed at a given locus.

The polymorphism index of individual or combined VNTR loci was calculated by use of the Hunter-Gaston discriminatory index (HGDI) (15), an application of Simpson' s index of diversity (36). This index evaluates the probability that two unrelated strains will be characterized as the same type. It is determined by the number of types defined by the test method and the relative frequencies of these types. The authors suggest that an index of greater than 0.90 is desirable if typing results are to be interpreted with confidence.

The MLVA genotype of an isolate with 15 VNTRs (MLVA15) is expressed as its allelic profile corresponding to the number of repeats at each VNTR in the order ms77, ms127, ms142, ms172, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, ms223, ms207, and ms209. The genotype of PAO1 deduced from its genomic sequence is 4 8 7 12 5 9 5 3 4 3 2 2 4 7 6. The genotype of strain PA14 deduced from its genomic sequence is 2 9 1 12 2 4 1 5 2 1 5 2 4 5 6. A new genotype number is given when one difference is observed at any VNTR. Lin-

TABLE 1. VNTR characteristics

Locus name	Position (kb) in PAO1	Primer name	Primer sequence	Repeat unit size (bp)	Product size bp (repeat copy no.)		No. of observed alleles (alleles)[a]	HGDI index[b]
					PAO1	PA14		
ms77	2263	ms77L ms77R	GCGTCATGGTCTGCATGTC TATACCCTCTTCGCCCAGTC	39	442 (4)	364 (2)	7 (2–6 + 1.5, 2.5)[c]	0.51
ms127	3496	ms127L ms127R	CTCGGAGTCTCTGCCAACTC GGCAGGACAGGATCTCGAC	15	210 (8)	225 (9)	2 (8–9)	0.33
ms142	3876	ms142L ms142R	AGCAGTGCCAGTTGATGTTG GTGGGGCGAAGGAGTGAG	115	890 (7)	201 (1)	9 (1–7 + 2.5, 3.5)	0.81
ms172	5084	ms172L ms172R	GGATTCTCTCGCACGAGGT TACGTGACCTGACGTTGGTG	54	789 (12)	789 (12)	6 (8, 10–13 + 8.5)	0.73
ms211	264	ms211L ms211R	ACAAGCGCCAGCCGAACCTGT CTTCGAACAGGTGCTGACCGC	101	663 (5)	360 (2)	8 (2–8 + 1.5)	0.76
ms212	521	ms212L ms212R	TGCTGGTCGACTACTTCGGCAA ACTACGAGAACGACCCGGTGTT	40	522 (9)	324 (4)	10 (3–9, 11–12, 14)	0.75
ms213	2568	ms213L ms213R	CTGGGCAAGTGTTGGTGGATC TGGCGTACTCCGAGCTGATG	103	640 (5)	221 (1)	7 (3–7, 9 + 4.5)	0.85
ms214	2705	ms214L ms214R	AAACGCTGTTCGCCAACCTCTA CCATCATCCTCCTACTGGGTT	115	426 (3)	655 (5)	4 (3–6)	0.81
ms215	4376	ms215L ms215R	GACGAAACCCGTCGCGAACA CTGTACAACGCCGAGCCGTA	129	765 (4)	507 (2)	7 (1–6 + 2.5)	0.80
ms216	4528	ms216L ms216R	ACTACTACGTCGAACACGCCA GATCGAAGACAAGAACCTCG	113	543 (3)	315 (1)	4 (1–4)	0.64
ms217	6187	ms217L ms217R	TTCTGGCTGTCGCGACTGAT GAACAGCGTCTTTTCCTCGC	109	606 (2)	933 (5)	7 (1–6 + 1.5)	0.79
ms222	5361	ms222L ms222R	AGAGGTGCTTAACGACGGAT TGCAGTTCTGCGAGGAAGGCG	101	390 (2)	391 (2)	7 (1–6 + 1.5)	0.76
ms223	5455	ms223L ms223R	TTGGCAATATGCCGGTTCGC TGAGCTGATCGCCTACTGG	106	454 (4)	453 (4)	7 (2–7 + 1.5)	0.77
ms207	2735	ms207L ms207R	ACGGCGAACAGCACCAGCA CTCTTGAGCCTCGGTCACT	6	146 (7)	134 (5)	12 (4–14, 17)	0.89
ms209	4541	ms209L ms209R	CAGCCAGGAACTGCGGAGT CTTCTCGCAACTGAGCTGGT	6	148 (6)	148 (6)	7 (4–10)	0.77

[a] Intermediately sized alleles are designated by "0.5" values to reflect this fact.
[b] Calculated with only one representative strain from each of the 64 genotypes.
[c] All alleles from 2 to 6, plus two intermediately sized alleles, called 1.5 and 2.5, were observed.

eages are defined as groups of isolates for which the genotype differs at a maximum of two VNTRs. In the clustering analysis by use of the categorical coefficient (also called Hamming's distance), this corresponds to an interval of 85 to 100% similarity. Lineages have been arbitrarily numbered in the order in which they are listed in the clustering analysis.

Nucleotide sequence accession numbers. The sequences of the alleles of unexpected sizes have been deposited in the EMBL sequence database under accession numbers AM773730 to AM773750.

RESULTS

Identification of new VNTRs for MLVA and analysis of stability. Since the initial attempts to define VNTRs for MLVA by using the sequence of the PAO1 strain (27), the genome of the *P. aeruginosa* strain PA14 was released and allowed the identification of new polymorphic tandem repeats by use of the program for strain comparison described by Denoeud and Vergnaud (8) (Table 1). In particular, a family of intergenic sequences which is abundantly distributed throughout the genome and whose members can be tandemly duplicated was found. These sequences are approximately 100 bp long, and they have about 90% identity. Nine such polymorphic sequences were selected for genotyping: ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, and ms223. The previously published marker ms142 (27) belonged to this family. This marker and three other minisatellites also described in the earlier study, minisatellites ms77, ms127, and ms172, were retained for the present investigation (the last four VNTRs described previously [27] were not used because they are too highly variable [ms10 and ms61] or proved to be too difficult to

TABLE 2. Genotypes of reference strains PAO1 and PA14 and additional characteristics of 24 isolates from 15 patients in the ICU

Patient	Strain	Alias	Date (day/mo/yr)[a]	Serotype	Source[b]	Lineage	Allele at the following ms VNTR marker:													
							77	127	142	172	211	212	213	214	215	216	217	222	223	207	209
	PAO1					36	4	8	7	12	5	9	5	3	4	3	2	2	4	7	6
	PA14					51	2	9	1	12	2	4	1	5	2	1	5	2	4	5	6
ICU-01	TR_S0400239	Tr21	02/02/2004	P11	TR	52	4	9	2	12	2	12	5	3	2	1	2	2	2	9	5
ICU-02[c]	TR_S0400578	Tr20	20/03/2004	P6	S	9	2.5	8	4	11	5	9	4	**2**	1	2	4	2	2	4	6
ICU-03	TR_S0400669	Tr18	01/04/2004	P6	TR	9	2.5	8	4	11	5	9	4	**2**	1	2	4	2	2	4	6
ICU-04	TR_S0401054	Tr14	01/06/2004	P6	S	9	2.5	8	4	11	5	9	4	**3**	1	2	4	2	2	4	6
ICU-05	TR_S0401618	Tr25	13/08/2004	P11	BL	32	3	8	1	13	4	6	3	2	2	1	3	1	3	6	4
ICU-06	TR_S0401805	Tr22	13/09/2004	P10	TR	49	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-06	TR_S0402010	Tr23	11/10/2004	P10	TR	37	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-06	TR_S0500094	Tr24	13/01/2005	P10	TR	37	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-06	TR_S0500094	Tr29	13/01/2005	P10	TR	49	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-06	TR_S0500240	Tr30	03/02/2005	P10	TR	37	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-06	TR_S0500684	Tr31	31/03/2005	P10	TR	37	3	9	4	11	3	9	5	2	1	2	2	6	NA	8	3
ICU-07	TR_S0401984	Tr16	05/10/2004	NT[d]	TR	28	2.5	8	1	11	6	9	9	2	3	1	3	3	3	8	2
ICU-07	TR_S0402049	Tr17	14/10/2004	P2	TR	33	2.5	8	1	11	6	9	9	2	3	1	3	3	3	8	2
ICU-08	TR_S0402104	Tr26	23/10/2004	P6	BL	37	2.5	8	6	12	2	NA	3	5	3	3	2	4	2	9	2
ICU-09	TR_S0402215	Tr15	12/11/2004	P11	S	43	6	9.5	1	10	2	9	5	4	2	1	1	2	4	11	4
ICU-10	TR_S0402497	Tr19	27/12/2004	P11	TR	2	2	8	3.5	11	1.5	7	1	2	1	IS	4	1	2	4	6
ICU-11	TR_S0500027	Tr27	05/01/2005	P11	EY	18	4	8	1	12	3	9	2	4	1	1	4	2	2	8	2
ICU-12	TR_S0500172	Tr28	24/01/2005	P3	S	7	2.5	8	7	12	3	3	8	3	1	2	1	1	2	4	2
ICU-13	TR_S0500408	Tr35	22/02/2005	NT	TR	27	3	8	2.5	11	6	6	1.5	6	5	2	3	3	3	9	2
ICU-13	TR_S0500407	Tr36	22/02/2005	NT	BL	31	3	8	2.5	11	6	6	1.5	6	5	2	3	3	3	9	2
ICU-13	TR_S0500413	Tr37	22/02/2005	NT	CRL	27	3	8	2.5	11	6	6	1.5	6	5	2	3	3	3	9	2
ICU-14	TR_S0500512	Tr32	09/03/2005	NT	TR	38	2.5	8	4	11	2	9	3	5	5	2	3	3	7	9	3
ICU-14	TR_S0500652	Tr33	27/03/2005	NT	S	42	2.5	8	4	11	2	9	3	5	5	2	3	3	7	9	3
ICU-15	TR_S0500586	Tr34	18/03/2005	P6	TR	19	2.5	8	4	11	3	8	2	5	6	2	4	3	2	8	2

[a] Date of strain isolation.
[b] TR, tracheal specimen; BL, blood; S, sputum; EY, eye; CRL, cephalorachidian liquid.
[c] The boldface highlights the three patients with strains of the same genotype except at ms214.
[d] NT, nontypeable.

type for routine use [ms173, large allele size range; ms194, small repeat unit compared to the allele sizes]). The polymorphisms of all these markers can be analyzed on a 2% agarose gel. We also included two new microsatellites, ms207 and ms209, with 6-bp repeat units. We used reference strain PAO1 as a control for the stability of these markers. PCR products of the expected sizes were found in this strain, suggesting that the culture conditions do not induce instability at a significant rate. To check that the new VNTRs could be used in a MLVA scheme, 50 isolates representative of the major genetic groups previously analyzed by Onteniente et al. (27) were typed (see Figure S1 in the supplemental material). The clustering obtained was compatible with that observed by two other genotyping techniques, PFGE and ribotyping. Although ms212 belongs to the family of intergenic repetitive elements with a repeat unit of about 100 bp, the observed PCR products are compatible with the duplication of a smaller unit about 40 bp long. This has been confirmed by sequencing several alleles.

As a first test of efficiency for the MLVA15 scheme (MLVA with 13 minisatellites and 2 microsatellites), we genotyped isolates from patients in the ICU panel (Table 2). Only nonmucoid bacteria were isolated from this panel; they were agglutinable in most of the cases and could therefore be serotyped. Amplification with the 15 markers was observed with all the isolates except for ms223 from patient ICU-06 and ms212 from patient ICU-08 (Table 2). When several isolates were recovered from a single patient, they showed the same MLVA profile, as illustrated for the six isolates recovered over a 6-month period from patient ICU-06, the three isolates from patient ICU-13, and the two isolates from patients ICU-07 and ICU-14. Isolates Tr20, Tr18, and Tr14, from patients ICU-02, ICU-03, and ICU-04, respectively, had the same serotype (P6) and an identical genotype except for ms214, suggesting that these patients were infected by variants of the same strain. They were present in the hospital within a period of 2 months. The other patients had unique strains with large genotype differences. In isolate Tr19 from patient ICU-10, the ms216 amplification product was about 1.5 kb, which is unusually large. Sequencing showed the presence of an insertion sequence (IS) in the tandem repeat (see the dedicated paragraph below). Each lineage was named according to the criteria defined in the Materials and Methods section.

Specificity of MLVA assay. In order to test the specificity of the VNTR amplifications, we analyzed 9 isolates belonging to three species frequently isolated from CF patients, *A. xylosoxidans*, *B. cepacia*, and *S. maltophila*, and 12 isolates from seven different environmental *Pseudomonas* species. No significant amplification signal was observed for the majority of VNTRs. Amplicons were sometimes obtained with ms77 and ms127, but they clearly had lower intensities compared to those of the *P. aeruginosa* bands (data not shown).

Longitudinal analysis of *P. aeruginosa* infection in CF patients. We then analyzed systematically all the *P. aeruginosa* isolates recovered from the CF patients over a period of 3 years (Table 3). They were typed by MLVA15 and, together with the ICU isolates and reference strains PAO1 and PA14, were grouped according to genotype after a clustering analysis was performed with BioNumerics software by use of Hamming's distance (the categorical coefficient) and the unweighted pair group method with arithmetic means clustering method (see Figure S2 in the supplemental material). Among the 163 *P. aeruginosa* isolates from 46 CF patients, 39 lineages were observed (Table 3). Seven lineages were shared by two different patients (lineages 11, 23, and 51), three different

TABLE 3. Characteristics of isolates from CF patients

Patient	Age (yr)[a]	Date (day/mo/yr) of isolation of:		No. of isolates[b]	Lineage	Genotype	Strain identifier[d]	Serotype
		First isolate	Last isolate					
CFU-01	21	14/01/2004		1 (1-0)	15	21	TR_S0400130	PNT
CFU-02[c]	6	13/02/2004		1 (1-0)	3	4	TR_S0400333	PNT
CFU-03	18	14/04/2004		2 (1-1)	25	35	TR_S0400738	PMU
CFU-04	10	21/06/2004	08/02/2005	2 (2-0)	25	35	TR_S0401260	PNT
CFU-05	16	09/06/2004	17/11/2006	9 (2-7)	21	28	TR_S0401093	PNT
CFU-05		12/07/2006		1 (1-0)	48	63	TR_S0601881	PNT
CFU-06	19	02/09/2004	14/03/2005	2 (0-2)	26	37	TR_S0401747	PMU
CFU-06		10/11/2004	23/03/2005	4 (4-0)	24	34	TR_S0402211	PNT
CFU-07	5	03/09/2004	01/06/2006	4 (4-0)	25	35	TR_S0401758	PNT
CFU-07		14/03/2005		1 (1-0)	44	58	TR_S0500554	PNT
CFU-08[c]	17	14/09/2004		2 (P11-0-1)	29	42	TR_S0401817	P11
CFU-09	19	06/10/2004	11/10/2006	14 (8-6)	1	2	TR_S0601070	PNT
CFU-09		07/12/2005		1 (1-0)	46	61	TR_S0502997	PNT
CFU-10[c]	7	05/01/2006	17/05/2006	6 (2-4)	13	19	TR_S0600035	PMU
CFU-11	18	19/01/2005	30/08/2006	7 (7-0)	17	24	TR_S0502246	PNT
CFU-12	18	21/01/2005	13/06/2006	8 (4-4)	4	5	TR_S0500158	PNT
CFU-13	10	02/02/2005	09/02/2006	11 (P11-10-0)	23	32	TR_S0500225	PNT
CFU-13		08/07/2005		1 (1-0)	16	22	TR_S0501523	PNT
CFU-14	20	28/02/2005	08/03/2006	2 (1-1)	12	17	TR_S0500464	PMU
CFU-15	18	13/04/2005	28/08/2006	6 (5-1)	10	14	TR_S0500773	PNT
CFU-16[c]	10	20/04/2005		1 (1-0)	51	67	TR_S0500826	PNT
CFU-17	8	04/05/2005	23/08/2006	6 (6-0)	1	1	TR_S0600287	PNT
CFU-18	12	22/06/2005	19/12/2005	2 (0-2)	21	28	TR_S0501374	PMU
CFU-19[c]	2	30/06/2005		1 (P3-0-0)	23	32	TR_S0501420	P3
CFU-20[c]	14	05/07/2005		1 (0-1)	11	16	TR_S0501497	PMU
CFU-21	11	13/07/2005		1 (1-0)	25	35	TR_S0501556	PNT
CFU-22	11	20/07/2005	26/07/2005	2 (P3-0-0)	42	56	TR_S0501638	P3
CFU-23[c]	2	10/09/2005	04/01/2006	3 (P3-1-0)	11	15	TR_S0502106	P3
CFU-24	7	14/09/2005	27/12/2006	7 (P3-P6-5-0)	45	59	TR_S0502141	P6
CFU-25	17	21/09/2005		1 (1-0)	5	6	TR_S0502224	PNT
CFU-26	10	27/09/2005	19/09/2006	3 (P6-2-0)	3	4	TR_S0502271	P6
CFU-27[c]	17	28/09/2005		1 (P5-0-0)	1	1	TR_S0502283	P5
CFU-28	18	07/10/2005	06/09/2006	5 (P11-4-0)	20	27	TR_S0502380	PNT
CFU-29[c]	14	02/11/2005		2 (1-1)	22	31	TR_S0502644	PMU
CFU-30	18	02/11/2005	25/04/2006	2 (2-0)	3	4	TR_S0502643	PNT
CFU-31	14	30/11/2005	12/10/2006	3 (0-3)	53	69	TR_S0502949	PMU
CFU-32	16	14/12/2005	20/12/2006	3 (0-3)	6	7	TR_S0503082	PMU
CFU-33[c]	3	26/01/2006	08/03/2006	2 (P3-1-0)	21	29	TR_S0600208	P3
CFU-33[c]		24/05/2006		1 (P11-0-0)	33	46	TR_S0601341	P11
CFU-34	6	21/02/2006	20/10/2006	7 (5-2)	40	53	TR_S0600483	PMU
CFU-35[c]	2	23/01/2006		1 (P4-0-0)	50	66	TR_S0600196	P4
CFU-36	11	22/02/2006	28/08/2006	5 (P6-4-0)	3	4	TR_S0600490	PNT
CFU-37[c]		27/03/2006		2 (P11-0-1)	14	20	TR_S0600819	P11
CFU-38	14	18/05/2006		1 (P10-0-0)	51	67	TR_S0601286	P10
CFU-39	17	29/06/2006		1 (0-1)	34	47	TR_S0601710	PMU
CFU-40[c]	19	03/07/2006	19/07/2006	2 (P4-1-0)	30	43	TR_S0601768	P4
CFU-41[c]	14	16/08/2006		1 (P11-0-0)	47	62	TR_S0602211	P11
CFU-42[c]	6	31/08/2006		1 (P11-0-0)	39	52	TR_S0602341	P11
CFU-43		25/09/2006	16/10/2006	2 (2-0)	41	54	TR_S0602586	PNT
CFU-44		05/10/2006	11/12/2006	4 (2-2)	8	10	TR_S0602698	PMU
CFU-45[c]	13	06/12/2006		1 (P6-0-0)	31	44	TR_S0603450	P6
CFU-46[c]	15	18/12/2006		1 (1-0)	35	48	TR_S0603599	PNT

[a] Age at onset of study.
[b] The designations and values in parentheses indicate occasional serotypes-number of PNT isolates-number of PMU isolates.
[c] Primary colonization.
[d] One representative strain.

patients (lineages 1 and 21), and up to four different patients (lineages 3 and 25); but a single lineage was commonly found in a patient, either with a nonmucoid (O-agglutinable or non-agglutinable) or a mucoid phenotype. Six patients harbored two different strains: CFU-05 (lineages 21 and 48), CFU-06 (lineages 26 and 24), CFU-07 (lineages 25 and 44), CFU-09 (lineages 1 and 46), CFU-13 (lineages 23 and 16), and CFU-33 (lineages 21 and 33). In four cases, one of these lineages was shared with other patients (lineages 1, 21, 23, and 25). For patients CFU-19, CFU-27, and CFU-33, a serotypeable strain corresponding to lineages 23, 1, and 21, respectively, was isolated at primary colonization, while nonserotypable (mucoid or nonmucoid) isolates from these lineages chronically infected several other patients. Interestingly, one strain from patient

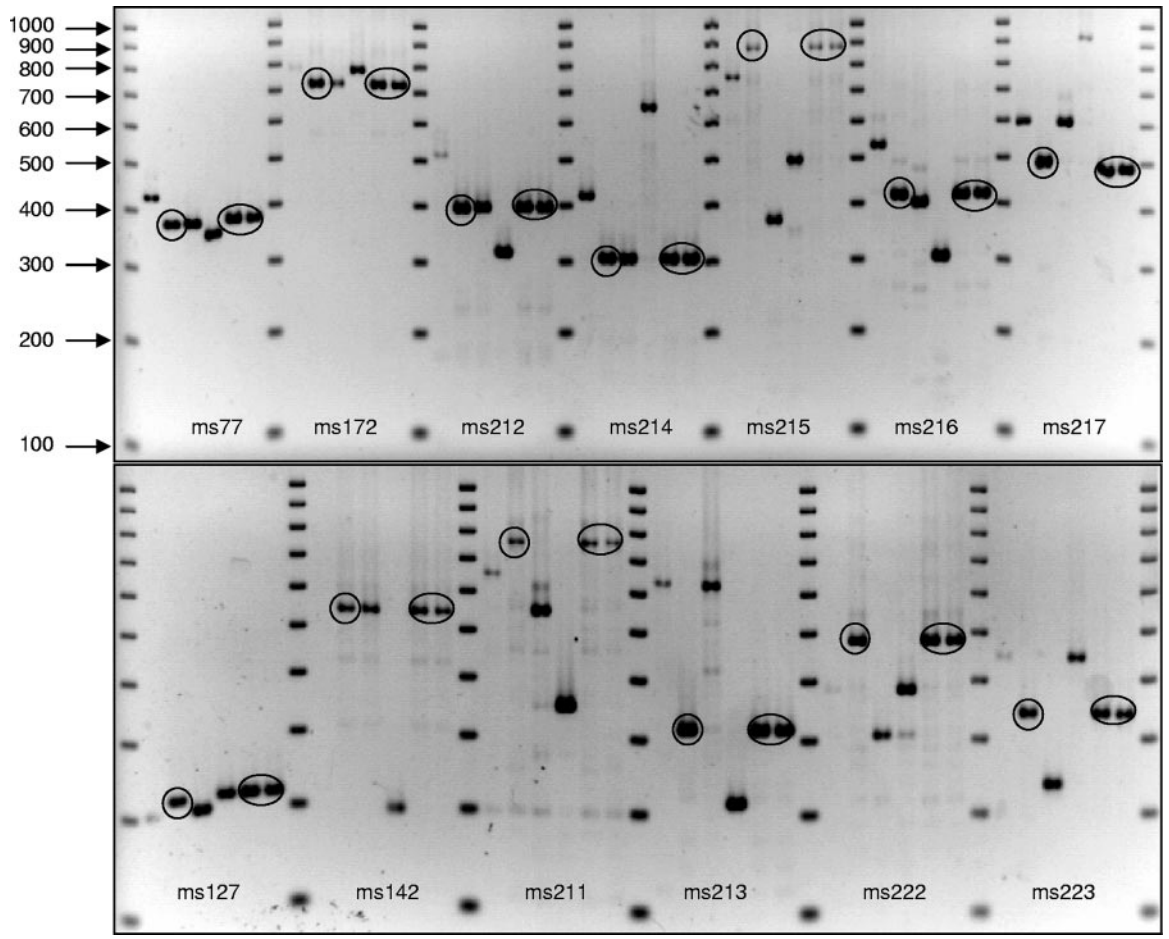

FIG. 1. Agarose gel electrophoresis of amplicons from 13 VNTRs in six *P. aeruginosa* isolates. Each group of six samples corresponds to strains PAO1, SG17, Tr42, Tr60, C50, and Tr40, respectively, and is separated by a 100-bp ladder. The amplicons in strains SG17, C50, and Tr40 are circled. The numbers on the left are in base pairs.

CFU-06 belonged to the same lineage (lineage 51) as two isolates belonging to clone C described by Römling et al. (34), isolates SG17M and C50, as shown in Fig. 1 (lanes 2, 5, and 6). The strain from patient CFU-06 differed from the other two strains only at ms207, one of the microsatellites. Similarly, one isolate from patients CFU-16 and CFU-38 had the same genotype as that of reference strain PA14, as deduced from the sequenced genome (20) (TR_S0500826, alias Tr60, from patient CFU-16 is shown in Fig. 1, lane 4).

Frequent insertion of ISPa11 into intergenic repeated elements. Alleles from 1.35 kb to more than 1.5 kb long were occasionally observed for minisatellites ms142, ms212, ms213, ms214, ms215, ms216, and ms223, suggesting the presence of an inserted element. A faint band was usually obtained upon amplification with longer elongation steps. At least one amplicon for each VNTR was sequenced, and the presence of an IS inserted into the intergenic element was shown in all the cases. In four cases a sequence corresponding to ISPa11 (28) was found, and in one instance (TR_S0502644, alias Tr66, at the ms214 locus) an IS similar to IS*222* was observed. Amplification of ms142 in Tr94 produced a 1.35-kb insert corresponding to the insertion of a sequence with no significant hit in the databases (GenBank release number 158). In lineage 23 an IS element was found in ms212 in one isolate (TR_S0500756, alias Tr56) from patient CFU-13 (but not in the other isolates of this lineage from the same patient) and from patient CFU-19.

DISCUSSION

Selection of a set of VNTRs for an efficient MLVA scheme. A major aspect of this work was to assign to each isolate a code reflecting its genetic profile, to store this information in a database, and to make interstrain comparisons. For this we extended our preliminary MLVA scheme by adding new VNTRs and by improving the purification of genomic DNA from alginate-producing isolates and the PCR efficiency. Indeed, with correctly purified DNA, good PCR amplification can be achieved in the presence of betain, which prevents the effect of the high G+C content. Among all the *P. aeruginosa* isolates tested, a PCR product was obtained for all the markers except for some null alleles, which were repeatedly observed with several DNA batches with the intergenic VNTRs (proportion of missing data, 2.5%). The absence of amplification was not explained but may have been due to the deletion of part or all of the element or locus. These null alleles were most

frequently observed with minisatellite ms213. Another amplification problem was due to the presence of an IS element in some markers, making the amplicon larger than 1.5 kb. Overall typing of the 15 selected markers was obtained for the majority of samples. For an easier and more robust MLVA scheme, the number of markers can be reduced to 14 (12 minisatellites and 2 microsatellites) by excluding ms213 or even to 10 (minisatellites ms142, ms211, ms212, ms213, ms214, ms215, ms216, ms217, ms222, and ms223). Indeed, a study by Johansson et al. with 10 VNTRs and 58 Swedish isolates from CF patients that had previously been genotyped by PFGE demonstrates that these isolates are similarly clustered by the two techniques (Ewa Johansson, personal communication). The two microsatellites ms207 and ms209 have high indices of diversity, reflected by an HGDI value close to or greater than 0.8 (Table 1), but their analysis requires the use of a sequencer or other high-resolution equipment. The global index of diversity for the 15 markers calculated with 190 isolates is 0.97%.

In this study we analyzed isolates from CF patients, as well as non-CF patients with different diseases caused by nosocomially acquired *P. aeruginosa* infections, in the Armand Trousseau Hospital (ICU patients) as well as in different European hospitals (27). The *P. aeruginosa* isolates from the CF and the non-CF patients were not distributed differently in the clustering analysis, although clones which chronically infected several CF patients were not found elsewhere.

The specificity of the assay was demonstrated by testing isolates from seven other *Pseudomonas* species as well as bacteria from other genera frequently found in the airways of CF patients (19). This showed that amplification of the 15 VNTRs could be obtained only with *P. aeruginosa* isolates. Furthermore, on two occasions during this study, isolates that could not be genotyped by MLVA15 turned out to be *Pantoea* spp. that had been misidentified as *P. aeruginosa.*

Stability of the VNTRs. The MLVA genotype of the strains recovered from an individual patient over time is remarkably stable, except for the occasional insertion of an IS element and the addition or deletion of repeats in a single VNTR, generally a microsatellite. For example, among seven isolates from patient CFU-05 that were analyzed (from 9 June 2004 to 4 January 2006), the last one differed from the others at microsatellites ms207 and ms209 by the loss of one repeat. Ten isolates from patient CFU-13 were analyzed from 2 February 2005 to 9 January 2006, and the isolates showed the same genotype, except for the presence of an IS in marker ms212 in one of the isolates.

Most of the VNTRs belong to a family of intergenic elements that can form hairpin secondary structures reminiscent of those of regulatory sequences such as the bacterial interspersed mosaic elements described in *Escherichia coli* (2). The presence of an IS and, in particular, the presence of ISPa11 was observed at least once in all these markers. A search of the PAO1 sequence showed the presence of this IS in additional elements not included in our MLVA scheme (40). It has previously been shown that intergenic repeated sequences were the target for the insertion of IS in *Pseudomonas putida* (33) as well as several other bacteria (40).

Longitudinal study of *P. aeruginosa* colonization. Treatment of most of the patients with primary colonization with combinations of antimicrobial agents seemed to be efficient, since *P. aeruginosa* was not isolated by our routine culture procedure several months after treatment (see Table S1 in the supplemental material). In 7 patients, the genotype of the strain causing the primary colonization was similar to the genotype associated with chronic colonization in other patients. Three patients (patients CFU-19, CFU-27, and CFU-33) were newly colonized with a serotypeable (agglutinable) strain that showed the same genotype as nonagglutinable (mucoid or nonmucoid) strains from chronically infected patients. This may be explained by a reversion of the nonagglutinable phenotype or by the existence of a reservoir of nonmucoid bacteria. A similar observation was made by Jelsbak et al., who further demonstrated that nonmucoid isolates in newly colonized patients were mucoid revertants (16).

Most of the CF patients were already colonized at the start of the present study, and they usually harbored a single strain, which persisted over an 18- to 24-month period, despite antibiotic treatment. Both mucoid and nonmucoid variants with the same genotype were isolated, as has been observed previously in CF patients (4, 24).

Are some lineages particularly prone to colonization of CF patients? Almost half of the CF patients were infected with strains whose VNTR patterns were highly similar to those of strains found in other CF patients. Strains with VNTR patterns identical to that of the PA14 reference strain (at the 15 markers tested) were isolated from two newly colonized patients (patients CFU-16 and CFU-38, lineage 51) and from another non-CF patient with primary ciliary dyskinesia attending the Trousseau CF Center (data not shown). In the three cases, the isolates were sensitive to several antibiotics, and infection was cleared following treatment. Strain PA14 was originally isolated from a burn patient and has been shown to be highly virulent for plants and animals (32). We further confirmed the identity between these isolates and PA14 by sequencing a particular tandem repeat structure, the CRISPR locus (31), which is present in some strains of *P. aeruginosa* and which is characterized by an important degree of variability (data not shown). The CRISPR sequence in the three isolates was exactly the same as that in PA14, whereas a high degree of diversity was observed in nonrelated isolates (data not shown). Another argument for the high degree of similarity between these isolates and PA14 is the presence of the pathogenicity island PAPI-1 (13), as assessed by PCR amplification of the RL038 and RL078 genes (data not shown). The basis for the pathogenicity of this strain is not known. Recent investigations suggested that its virulence may be multifactorial and combinatorial (20). The VNTR pattern of lineage 26 corresponds to that of clone C, another lineage that is distributed worldwide and that is frequently observed in CF patients. These clones of pathogenic *P. aeruginosa* may infect patients other than those in CF clinics, suggesting that they are probably widely distributed in the environment. Finally, six additional lineages described in the present study have been identified in CF patients in Sweden (Ewa Johansson, personal communication).

Widespread clones of *P. aeruginosa* have previously been isolated from patients attending pediatric CF centers (1). The spread of particular clones was sometimes related to antibiotic resistance (6, 17). A large study in England and Wales suggested that some clones were distributed among different CF centers (35). A recent study in Denmark investigating *P.*

aeruginosa populations in CF patients also reported the existence of dominant clones that proved to be highly successful colonizers of the airways of CF patients (16).

Studies on the population structure of *P. aeruginosa* revealed the existence of clonal complexes in clinical and environmental isolates (29, 43). In the study by Wiehlmann et al. of 240 *P. aeruginosa* strains from diverse habitats and of diverse geographic origins, the majority of strains belonged to few dominant clones, and the most frequent genotype was represented by strain PA14 (43).

Since most of our patients seemed to have no contact with each other outside the CF center (the exceptions were two brothers, patients ICE-27 and ICU-45, who did not share the same strain), an epidemiological study will be performed to clarify the origins of apparent cross transmission of some *P. aeruginosa* lineages and the possibility of a common source of infection within the center or from an unidentified environmental source. It is not known whether these clones are more virulent than sporadic clones, and studies by use of, for example, the *Caenorhabditis elegans* killing assay will need to be performed (38).

Conclusion. This study demonstrates that MLVA is a very robust genotyping technique which can be applied to the systematic survey of *P. aeruginosa* isolates in a CF clinic. Future developments of the MLVA procedure can be achieved by using a sequencer in combination with different fluorochromes and loci with different PCR size ranges. An isolate could then be genotyped in a single run either by mixing the products after the PCR or by performing a multiplex PCR.

The characterization of germs that chronically infect CF patients is of major importance for improving the treatment of these patients. To this purpose, VNTR-based genotyping assays will be applied to the systematic analysis of other germs and in particular to *Staphylococcus aureus* and *Burkholderia* spp., for which specific MLVA schemes have been developed.

MLVA allows the comparison of isolates between different centers through exchanges of VNTR profiles. These profiles and associated lineages will be stored in databases. As such, we propose a genotyping page on a public genotyping website in which MLVA profiles can be compared to those of the present strain collection (http://bacterial-genotyping.igmors.u-psud.fr/). We believe that such tools will help address the problem of the worldwide distribution of pathogenic clones and investigation of the basis for their pathogenicity.

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